Kidney International, Vol. 61 (2002), pp. 502–515

TNF-α–accelerated apoptosis abrogates ANCA-mediated neutrophil respiratory burst by a caspase-dependent mechanism[1]

Ralph Kettritz, Julia Scheumann, YaXin Xu, Friedrich C. Luft, and Hermann Haller

HELIOS Klinikum-Berlin, Franz Volhard Clinic, and Max Delbrück Center for Molecular Medicine, Medical Faculty of the Charité, Humboldt University of Berlin, Berlin and Division of Nephrology, Department of Medicine, Hannover Medical School, Hannover, Germany

TNF-α-accelerated apoptosis abrogates ANCA-mediated neutrophil respiratory burst by a caspase-dependent mechanism.

Background. Tumor necrosis factor (TNF)-α rapidly primes neutrophils (PMN) for an anti-neutrophil cytoplasmic antibody (ANCA)–induced respiratory burst and is thus proinflammatory. TNF-α also progressively accelerates apoptosis. We investigated the effect of TNF-α-mediated apoptosis on ANCA antigen expression and on ANCA-induced superoxide generation in human PMN.

Methods. PMN were brought to apoptosis by 10 ng/mL of TNF-α or a combination of TNF-α and 2.5 μg/mL cycloheximide, a protein synthesis inhibitor, or cycloheximide alone for three hours. Apoptosis and ANCA antigen expression were assessed by fluorescence-activated cell sorting (FACS) and microscopy. Superoxide was determined with the ferricytochrome C assay.

Results. TNF-α with cycloheximide for three hours caused apoptosis in 87% PMN compared to 2% in untreated controls ($N = 18$; $P < 0.01$). Accelerated apoptosis was associated with an increase in ANCA-antigen expression for both proteinase 3 and myeloperoxidase ($P < 0.05$). Nevertheless, apoptosis was paralleled by a decreased proteinase 3 and myeloperoxidase ANCA-induced respiratory burst ($P < 0.05$). Furthermore, superoxide release in response to immune complexes, phorbol ester (PMA), and bacterial peptide (FMLP) was significantly decreased. Blocking caspase-3 activity prevented apoptosis in TNF-α with cycloheximide-treated cells (83% to 2%) and prevented compromised respiratory burst in response to ANCA. Caspase-3 inhibition abrogated apoptosis-mediated ANCA antigen up-regulation (PR3 141.6 ± 34.1 MFI to 33.9 ± 7.8; MPO 48.3 ± 12.9 MFI to 11.9 ± 3.2, $N = 6$, $P < 0.05$).

Conclusions. TNF-α–accelerated apoptosis was associated with increased ANCA antigen expression but with down-regulated respiratory burst activity in response to ANCA. Specific inhibition of apoptosis by caspase-3 blockade prevented the increase in ANCA-antigen expression and preserved the capability of generating superoxide, thereby establishing a causative role for apoptosis. We suggest that TNF-α exhibits dual actions by both priming and terminating ANCA-mediated activation of human PMN.

Anti-neutrophil cytoplasmic antibodies (ANCA) are found in patients with microscopic polyangiitis, Wegener's granulomatosis, Churg-Strauss syndrome, and pauci-immune necrotizing crescentic glomerulonephritis [1–4]. ANCA-activated neutrophils (PMN) may play an important role in the pathogenesis of the vasculitis. Tumor necrosis factor-α (TNF-α) is a cytokine that is up-regulated in active vasculitis [5–9]. TNF-α primes PMN for a consecutive ANCA-induced respiratory burst [10–12]. ANCA alone cannot stimulate a significant respiratory burst in resting PMN. We reported earlier that the priming process involves activation of mitogen-activated protein kinase (MAPK) pathways, whereby p38-MAPK controls the TNF-α-mediated increase in ANCA antigen translocation from the primary granules to the cell surface [13]. On the one hand, TNF-α–mediated priming is proinflammatory by increasing ANCA binding, resulting in full-blown neutrophil activation. On the other hand, TNF-α also may be antiinflammatory by accelerating early neutrophil apoptosis [14–16]. In earlier studies, apoptotic PMN showed a compromised respiratory burst to some stimuli, whereas an unchanged response was observed to other stimuli. Constitutive PMN apoptosis decreased superoxide generation after receptor-dependent stimuli, such as bacterial peptide (FMLP) and opsonized zymosan, with an unchanged response to PMA, a receptor-independent PMN activator [17]. Interestingly, constitutive apoptosis is associated with an increased ANCA-antigen expression on the cell membrane, allowing more ANCA to bind [18]. However, the effect on

[1] **See Editorial by Kallenberg, p. 758.**

Key words: PMN, anti-neutrophil cytoplasmic activity, tumor necrosis factor-α, cell death, respiratory burst, proinflammation.

Received for publication December 12, 2000
and in revised form September 24, 2001
Accepted for publication September 25, 2001

ANCA-mediated reactive oxygen species stimulation was not studied. Apoptosis-induced ANCA-antigen upregulation may allow more ANCA to interact with PMN, which would prevent or counteract apoptosis-mediated loss of respiratory burst activity. We tested the hypothesis that, in addition to its well-known early proinflammatory priming effect, TNF-α terminates ANCA-mediated PMN activation via apoptosis. Since TNF-α not only rapidly accelerates apoptosis, but also induces survival signals probably via activating nuclear factor-κB (NF-κB) transcription factors [19, 20], we combined TNF-α with cycloheximide to block these protein synthesis-mediated survival effects.

METHODS

Materials

Plasmagel was obtained from Zeptometrix Corporation (Buffalo, NY, USA) and Ficoll-Hypaque from Sigma (Deisenhofen, Germany). RPMI 1640, trypan blue, and PBS were obtained from Biomed (Berlin, Germany). Hank's balanced salt solution (HBSS) was from Biochrom (Berlin, Germany) and recombinant TNF-α from R&D Systems (Wiesbaden, Germany). Formyl-methionyl-leucyl-phenylalanine (FMLP), bovine erythrocyte superoxide dismutase (2500 to 7000 U/mg protein), ferricytochrome C, cytochalasin B, cycloheximide, phorbol myristate acetate (PMA), albumin (bovine fraction V), and rabbit-anti-BSA antibody were from Sigma. The polyclonal rabbit-antibodies to tyrosine phosphorylated ERK were acquired from New England Biolabs, Inc. (Beverly, MA, USA). The ApoAlert™ Annexin V Apoptosis Kit was from Clontech (Palo Alto, CA, USA). The irreversible inhibitor, preferentially targeting caspase-3 (Ac-DEVD-CMK) was purchased from Calbiochem (Bad Soden, Germany). Endotoxin-free reagents and plastic disposables were used in all experiments.

Isolation of human PMN and culture conditions

Polymorphoneuclear neutrophils from healthy human donors, patients with ANCA-vasculitis or disease controls were isolated from heparinized whole blood by red blood cell sedimentation with plasma gel, followed by Ficoll-Hypaque (Sigma) density gradient centrifugation. Erythrocytes were lysed by incubation with hypotonic saline for 15 seconds. PMN were spun down (200 × *g*, 7 min) and reconstituted in HBSS with calcium and magnesium ($HBSS^{++}$) or, when cultured for up to three hours in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, penicillin, and streptomycin. Ten microliters of neutrophil suspension were incubated with 40 μL trypan blue for five minutes at room temperature (RT). Cells were counted in duplicate using a hemocytometer and considered viable if able to exclude trypan blue. The cell viability was detected in every cell preparation and found to be greater than 99%. The percentage of PMN in the suspension was >95% by a Wright-Giemsa staining and by light microscopy. Apoptosis was induced by culturing cells in the presence of 10 ng/mL TNF-α, 2.5 μg/mL cycloheximide, a combination of TNF-α and cycloheximide, or buffer control, respectively. To block caspase-3 activity cells were preincubated in some experiments for 30 minutes with 50 μmol/L Ac-DEVD-CMK, the irreversible inhibitor of caspase-3. Samples were incubated in polypropylene tubes and kept at 37°C in 5% CO_2.

Preparation of immunoglobulins

Human IgG was prepared from patients with biopsy-proven Wegener's granulomatosis (3 PR3-ANCA) and microscopic polyangiitis (2 MPO-ANCA) as well as from two healthy controls as described recently [21]. Plasma samples were obtained from freshly drawn blood and kept at −20°C. Plasma was filtered through a 0.2 μm syringe filter (Gelman Sciences, Ann Arbor, MI, USA) and applied to a protein G affinity column (Pharmacia, Uppsala, Sweden). Bound immunoglobulins were eluted with 0.1 mol/L glycine-HCl buffer, pH 2.75 (elution buffer). After the antibodies emerged, the pH was immediately adjusted to pH 7.0 using 1 mol/L Tris-HCl, pH 9.0. A mouse monoclonal to MPO (MPO-7, IgG1κ) and an isotype-matched control (IgG1κ) were purchased from Dako (Hamburg, Germany). Prior to use, IgG preparations were centrifuged at 10,000 rpm for five minutes to remove aggregates.

Measurement of apoptosis using propidium iodide stained PMN and annexin V binding by flow cytometry

Flow cytometry was used to measure DNA content in Ethanol-permeabilized cells at the single cell level as described previously [15]. Briefly, freshly isolated or cultured cells were spun at 200 × *g* for five min at 4°C and resuspended in phosphate-buffered saline (PBS) containing 0.5 mmol/L ethylenediaminetetraacetic acid (EDTA). Chilled 95% ethanol was added to a final concentration of 70% and the cells were stored at −20°C for one to two days. PMN were pelleted (200 g, 5 min, 4°C) and resuspended in 250 μL PBS/0.5 mmol/L EDTA/1% bovine serum albumin (BSA). Then, 250 μL PBS containing 200 μg DNase-free RNase and 500 μL PBS containing 50 μg propidium iodide were added. After six to eight hours in the staining mixture at 4°C, PMN were analyzed using a fluorescence activated cell sorter (FACscan; Becton Dickinson, Heidelberg, Germany) and 10,000 events per sample were collected in list mode using Lysis II software for data acquisition and analysis. For annexin V staining, freshly isolated or cultured cells (5×10^5) were washed with PBS and pelleted, followed by resuspension in 200 μL binding buffer. Then, 10 μL

of annexin V were added to the cells and mixed gently. After incubation in the dark at RT for 10 minutes, the cells were subjected to flow cytometry analysis.

Cell staining with Wright Giemsa

Cytocentrifuge preparations of cells were fixed in 100% methanol and stained using modified Wright-Giemsa stain. Morphologic features of apoptosis as described by Kerr et al [22], including pyknotic nuclei, nuclear and cytoplasmic condensation, and the formation of apoptotic bodies were considered as evidence for apoptosis.

Measurement of necrosis using propidium iodide stained PMN by flow cytometry

Flow cytometry was used to assess necrosis in PMN cultured in the presence of 10 ng/mL TNF-α, 2.5 μg/mL cycloheximide, a combination of TNF-α and cycloheximide, or buffer control, respectively. After three hours cells were spun at 200 × *g* for five minutes at 4°C and resuspended in PBS containing 5 μg/mL propidium iodide. After 10 minutes in the staining mixture at room temperature, PMN were analyzed using a FACscan. A total of 10,000 events per sample were collected in list mode using Lysis II software for data acquisition and analysis. Using unfixed cells, positive staining with propidium iodide indicates loss of membrane integrity. These cells were considered to be necrotic.

Western blot analysis for phosphorylated ERK

Polymorphonuclear neutrophils were incubated at a concentration of 5×10^6/mL in the presence of 10 ng/mL TNF-α, 2.5 μg/mL cycloheximide, a combination of TNF-α and cycloheximide, or buffer control, respectively for 7 minutes, and 1, 2, and 3 hours, respectively. Samples were harvested and cell lysates prepared by resuspending cells in 20 μL of ice-cold lysing solution (20 mmol/L Tris-HCl, pH 8.0, containing 138 mmol/L NaCl, 1% Triton X-100, 2 mmol/L EDTA, 10% glycerol, 0.2 mmol/L sodium orthovanadate, 1 mmol/L PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 0.1 mmol/L quercetin, 5 mmol/L iodoacetamide). Samples were stored for 20 minutes on ice and centrifuged at 12,000 × *g* for five minutes at 4°C. Supernatant was recovered and the protein concentration was estimated by the BCA protein assay (Pierce, Munich, Germany). Samples were incubated for five minutes at 95°C in loading buffer (250 mmol/L Tris-HCl, pH 6.8, with 4% SDS, 20% glycerol, 0.01% bromphenol blue) and 25 μg protein per lane were loaded on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel, electrophoresed, and blotted onto polyvinylidene difluoride membrane by semi-dry technique. The membrane was blocked in 1% skim milk/0.05% Tween/PBS overnight at 4°C. Phosphotyrosine was detected using specific polyclonal rabbit antibodies to phospho-ERK1/2 (1000 dilution) and a horseradish peroxidase-labeled donkey anti-rabbit IgG (1:1000). Blot was developed by incubation in a chemiluminescence substrate (NEN Life Science Products) and exposed to x-ray film.

Superoxide generation assay

Superoxide was measured using the assay of superoxide dismutase (Sigma)-inhibitable reduction of ferricytochrome C as described by Pick and Mizel [23]. Briefly, freshly isolated PMN or PMN cultured for the indicated time periods were pretreated with 5 μg/mL cytochalasin B for 15 minutes at 4°C. Then, 0.75×10^6 cells were primed with 2 ng/mL TNF-α for 15 minutes at 37°C before anti-MPO monoclonal antibody (mAb) or human ANCA preparations were added. No priming was performed, when cells were stimulated with PMA, immune-complexes, or FMLP. The final concentrations were 2.5 μg/mL for the mAb to MPO, 75 μg/mL for purified IgG preparations, 25 ng/mL of PMA, 25 μg/mL immune complexes, and 10^{-7} mol/L of FMLP. All experiments were set up in duplicates. The samples were incubated in 96-well plates at 37°C for up to 120 minutes and the absorption of samples with and without 300 U/mL superoxide dismutase (SOD) was scanned repetitively at 550 nm employing a Microplate Autoreader. The final ferricytochrome C concentration was 50 μmol/L, and the final cell concentration was 3.75×10^6/mL. No activating effect was seen when human and mouse control antibodies were employed or when cells were primed with 2 ng/mL of TNF-α. The baseline activity of TNF-α–treated PMN was determined in every experiment and was factored for each condition.

Preparation of immune complexes

Immune complexes were prepared as described elsewhere [24]. The specific rabbit anti-BSA antibody was diluted in HBSS to a concentration at 1 mg/mL and BSA as its antigen at 0.25 mg/mL. Several antigen-antibody ratios were incubated at 37°C for two hours followed by overnight incubation at 4°C. Protein concentration was assessed to determine maximum immune complex formation. Based on this titration an antibody to antigen ratio of 4:1 was used for neutrophil stimulation. The final immune complex concentration in the superoxide assay was 25 μg/mL.

Assessment of ANCA-antigen expression by flow cytometry

Flow cytometry was used to evaluate the effect of apoptosis on PR3- and MPO-expression in PMN. Immunostaining was performed as described previously [21]. Briefly, cells were incubated at a concentration of 5×10^6/mL in the presence of 10 ng/mL TNF-α, 2.5 μg/mL cycloheximide, a combination of TNF-α and cyclohexi-

mide, or buffer control, respectively. After three hours, cells were pelleted at 200 g for five minutes at 4°C and resuspended in 1 mL ice-cold PBS/0.5% paraformaldehyde. After washing in HBSS without Ca^{2+}/Mg^{2+} cells were incubated with dilutions of mAb to PR3 (CLB 12.8, CLB, Amsterdam, Netherlands), MPO (MPO-7; Dako), or an isotype control followed by a secondary FITC-conjugated $F(ab)_2$-fragment of goat anti-mouse IgG. Flow cytometry was performed on the same day using a FACScan (Becton Dickinson), and 10,000 events per sample were collected. The mab to either PR3 or MPO, followed by a secondary PE-conjugated $F(ab)_2$-fragment of goat anti-mouse IgG were used for double staining. After ANCA antigen staining, cells were washed, resuspended in the proprietary binding buffer for annexin V, and incubated with FITC-conjugated annexin V for 10 minutes.

Statistical analysis

Results are given as mean ± SEM. Comparisons between two groups were done using paired Wilcoxon rank tests. Comparisons between multiple groups were done using Kruskal-Wallis tests. Specific differences between multiple groups were then determined by use of a Bonferroni post-hoc test on the ranked values. Ranked values were used because of the well-known interindividual variability in neutrophil responses.

RESULTS

Combined treatment with TNF-α and cycloheximide

We first established an apoptosis time course in normal human PMN treated with control buffer, 10 ng/mL TNF-α, a combination of 10 ng/mL TNF-α and 2.5 μg/mL cycloheximide, and 2.5 μg/mL cycloheximide, respectively. PMN from healthy donors were cultured for up to three hours and the percentage of apoptotic cells was determined at the indicated time points (Fig. 1A). The flow cytometry results in ethanol-fixed PI-stained cells show that TNF-α–induced progressive apoptosis, and that cycloheximide strongly potentiated this effect. Apoptotic cell death involved approximately 85% of PMN after three hours. Based on this time course, the three-hour time point was selected for further studies. A total of 18 experiments were performed assessing the effect of TNF-α, TNF-α with cycloheximide, and cycloheximide alone on PMN apoptosis at three hours in parallel. Figure 1B summarizes the results confirming the data of the time course study. Increased exposure of phosphatidyl serine on the outer cell membrane represents typical apoptosis surface changes. Flow cytometry was performed using annexin V binding. A representative of six experiments is given in Figure 2. Increased annexin V binding in samples treated with TNF-α and the combination of TNF-α with cycloheximide indicates that both conditions triggered the characteristic apoptotic surface

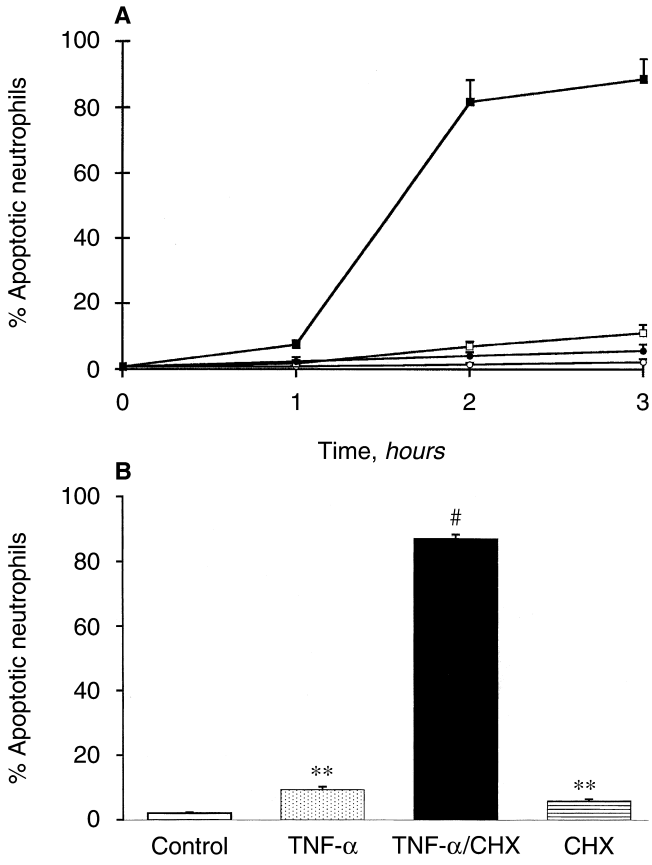

Fig. 1. Effects of buffer control (○), 10 ng/mL tumor necrosis factor-α (TNF-α; □), a combination of 10 ng/mL TNF-α and cycloheximide (TNF-α/CHX; ■), and CHX alone (●) on polymorphonuclear neutrophil (PMN) apoptosis as determined simultaneously. Apoptosis was assessed by flow cytometry assaying ethanol-fixed cells that were stained with propidium iodide (PI). To establish a time course, freshly isolated PMN were cultured up to three hours and the percentage of apoptotic cells was determined at the indicated time points (*A*, $N = 3$). (*B*) The percentage of apoptotic cells after 3 hours of culture ($N = 18$). The data indicate that TNF-α accelerates apoptosis in a time-dependent manner, and that CHX strongly potentiated TNF-α–induced apoptosis, involving approximately 90% of cells (**$P < 0.01$ compared to CTRL; #$P < 0.01$ compared to all other groups).

program. The percentage of apoptosis was 6 ± 1% in control cells, 16 ± 1% with TNF-α, 87 ± 2% with TNF-α/CHX, and 9 ± 2% with cycloheximide alone. In addition, the combined treatment caused the typical morphological signs of apoptosis as demonstrated by staining with modified Wright Giemsa (Fig. 3). These apoptotic features included pyknotic nuclei, nuclear condensation and formation of apoptotic bodies. Importantly, apoptosis at three hours was not accompanied by a loss of membrane integrity as shown by the ability of unfixed PMN to exclude propidium iodide using flow cytometry. The percentage of viable cells was >95% in all samples (99.0 ± 0.1% in control cells) excluded propidium iodide. These values were 98.7 ± 0.2% for treatment with TNF-α, 96.1% for the combined treatment, and 98.8 ±

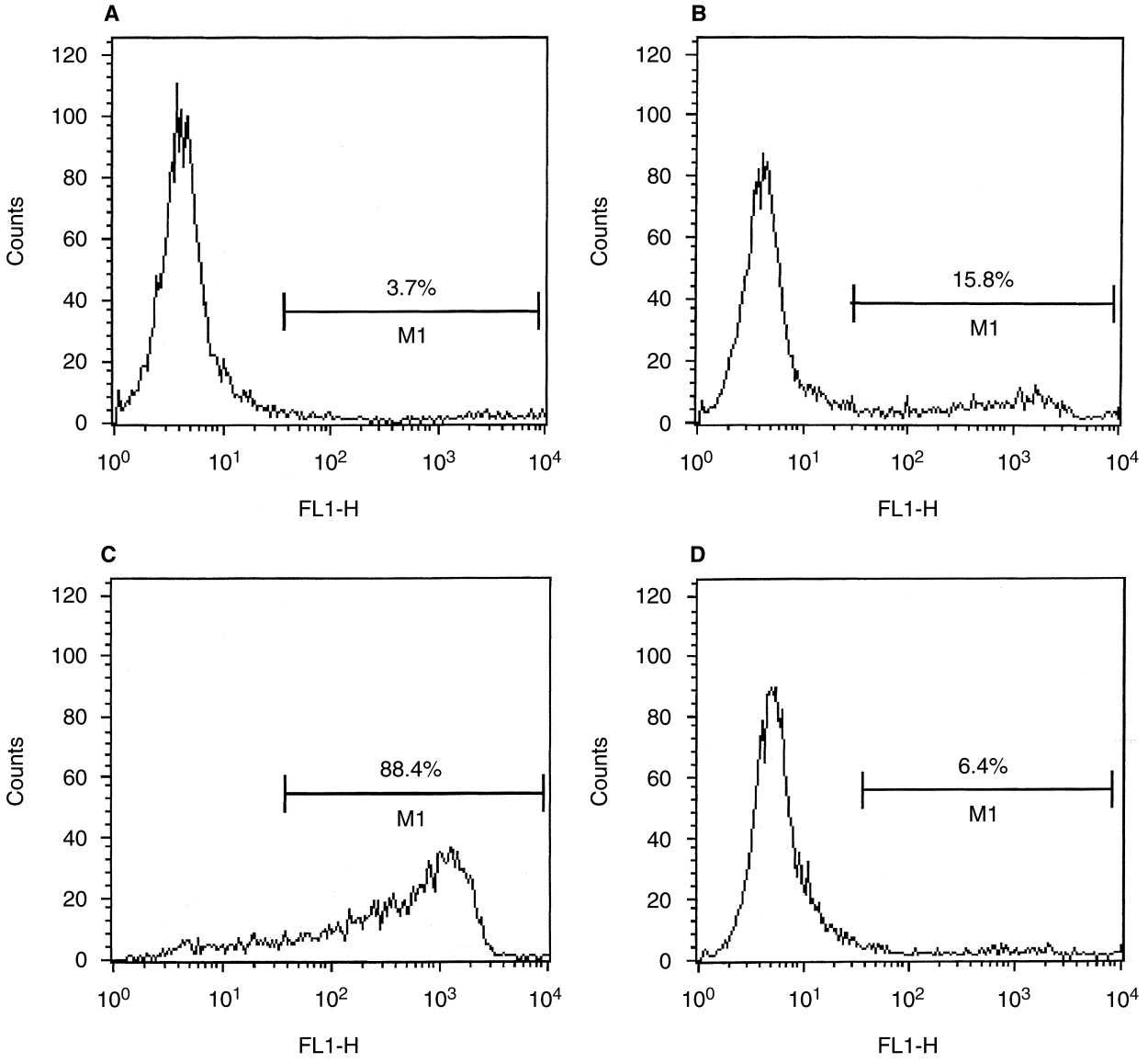

Fig. 2. Effects of buffer control (*A*), 10 ng/mL TNF-α (*B*), a combination of 10 ng/mL TNF-α and cycloheximide (*C*), and cyclohexamide alone (*D*) on PMN apoptosis was determined by flow cytometry using annexin V^{FITC}. A representative of 6 experiments is given. Samples treated with TNF-α (B) and the combination of TNF-α with cycloheximide (C) demonstrated increased annexin V binding, compared to control cells (A) and cycloheximide alone (D).

0.2% for cycloheximide alone, respectively; $N = 18$). Some members of the trichothecin mycotoxin family, such as cycloheximide activated ERK in a RAW and U937 cell line [25, 26]. Potential cycloheximide effects on ERK activation in human PMN could have affected functional responses, including respiratory burst, independent of apoptosis. To further validate the model used in our study, we investigated the effect of cycloheximide on ERK phosphorylation. PMN were incubated in control buffer, 2.5 μg/mL cycloheximide, 10 ng/mL TNF-α, and a combination of 10 ng/mL TNF-α and 2.5 μg/mL cycloheximide, respectively. Samples were harvested after 7 minutes, and 1, 2, and 3 hours, respectively, and Western blotting for phosphorylated ERK was performed. The seven-minute time point was included since our prior study found that TNF-α induced ERK phosphorylation peaked at this time point [13]. Figure 4 indicates that TNF-α results in a strong phosphorylation of ERK at seven minutes, and that cycloheximide either alone or combined with TNF-α had no effect during the three-hour incubation. Together, these data demonstrate that PMN challenged with a combination of TNF-α and protein synthesis inhibition with cycloheximide rapidly undergo apoptosis involving the vast majority of the cell population, whereas significant secondary necrosis was not detected up to three hours.

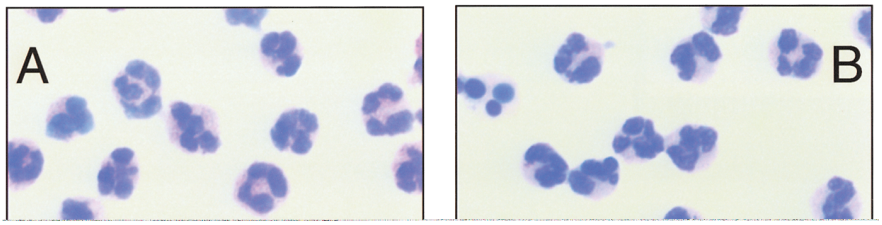

Fig. 3. PMN apoptosis after TNF-α 10 ng/mL, a combination of 10 ng/mL TNF-α and CHX, and CHX alone was determined. PMN were cultured for 3 hours. Cells were stained with Wright-Giemsa and apoptosis was assessed by microscopy. PMN cultured in buffer control (*A*) did not show apoptotis. TNF-α resulted in some apoptotic cells (*B*), with a dramatic acceleration by a combined TNF/CHX treatment (*C*). Only occasionally, apoptotic PMN were observed in the presence of CHX alone (*D*). Typical experiments are shown.

TNF-α–mediated apoptosis and membrane expression of ANCA-antigens

To study the effect of apoptosis on the membrane expression of ANCA antigens, PMN were cultured in control buffer, TNF-α, TNF-α with cycloheximide and cycloheximide alone for three hours. Membrane expression of PR3 and MPO was assessed by flow cytometry using specific mAb. There was an increase in the amount of expressed ANCA-antigens in PMN treated with TNF-α (Fig. 5). The combined treatment further potentiated this effect. Control cells showed a mean fluorescence intensity (MFI) for PR3 membrane expression of 36.5 ± 3.9 with an increase to 51.0 ± 5.8 in the TNF-α–treated sample ($P < 0.01$) and to 116.0 ± 15.2 in the TNF-α combined with cycloheximide sample ($P < 0.01$). Cycloheximide alone did not affect PR3 expression (Fig. 5A). MPO expression significantly increased from 20.8 ± 1.8 to 28.9 ± 3.5 when cells were cultured in the presence of TNF-α, and to 58.6 ± 8.2 with the combined treatment (Fig. 5B). As seen for PR3, MPO expression was not altered by cycloheximide alone. To study the relationship between TNF-α–induced apoptosis and ANCA antigen expression in more detail, we performed double staining for apoptosis and either PR3 or MPO, respectively. A representative experiment is given in Figure 6. The green fluorescence in FL-1 shows apoptosis by annexin V binding, and the red fluorescence in FL-2 detects membrane staining for the ANCA antigens. For PR3 the expected distribution of a positive and a negative population was distinguished. TNF-α increased both PR3 and MPO on the surface of non-apoptotic annexin V-negative PMN, confirming the well-known priming effect of TNF-α. Furthermore, TNF-α increased the number of annexin V-positive apoptotic cells, and more than doubled the percentage of apoptotic cells that stained positive for the ANCA antigens, compared to untreated controls (4.3 ± 1 to 10.8 ± 2% for PR3, and 2.8 ± 0.3 to 7.2 ± 0.3% for MPO; $N = 4$ for PR3 and $N = 3$ for MPO). At the same time, the percentage of ANCA antigen-negative non-apoptotic PMN declined (47.5 ± 4 to 39.1 ± 4% for PR3, and 90.1 ± 2 to 69.4 ± 6% for MPO). A further strong increase in the percentage of apoptotic ANCA antigen-positive cells was observed with combined treatment of TNF-α and cycloheximide; 54.1 ± 6 were positive for both apoptosis and PR3, while 23.8 ± 1 were positive for apoptosis and MPO. No effect of cycloheximide alone was found (data not shown). Moreover, comparison of PR3- and MPO-positive PMN within the TNF-α–stimulated sample revealed a higher expression of ANCA antigens on the surface of apoptotic cells, compared to the non-apoptotic population (901 ± 202 MFI vs. 446 ± 91 for PR3, and 797 ± 70 MFI vs. 221 ± 128 MFI for MPO). These data demonstrate that TNF-α-mediated apoptosis, in addition to TNF-α–mediated priming, is associated with an increased expression of ANCA-antigens.

TNF-α–mediated apoptosis and ANCA-induced respiratory burst

To test the effect of TNF-α–mediated apoptosis on ANCA-induced respiratory burst activity, first a mAb to MPO was used to establish a time course of superoxide

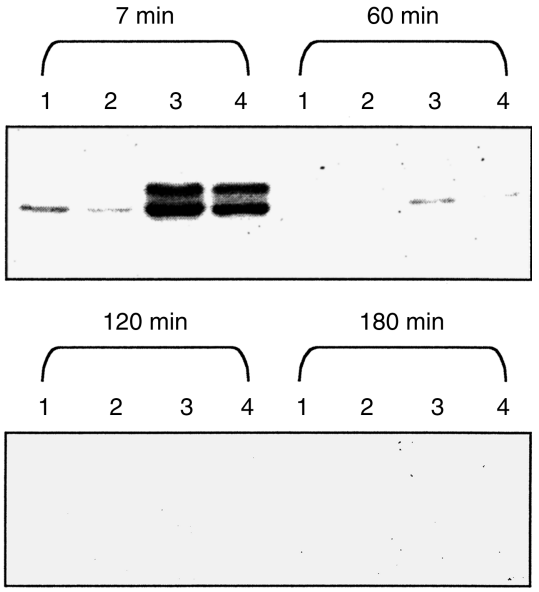

Fig. 4. Western blotting for ERK phosphorylation was performed in duplicate. PMN were incubated for up to 3 hours in control buffer (lane 1), 2.5 μg/mL cycloheximide (lane 2), 10 ng/mL TNF-α (lane 3), and a combination of 10 ng/mL TNF-α and CHX (lane 4). Samples were harvested after the indicated time points and phosphorylation of ERK was assessed. The data indicate that TNF-α activated ERK at 7 minutes. Cycloheximide did not affect ERK phosphorylation, either alone or when added in combination with TNF-α. The 120- and 180-minute time points indicate that the phosphorylation was short-term and in this 3-hour assay at no time point a function of cycloheximide presence.

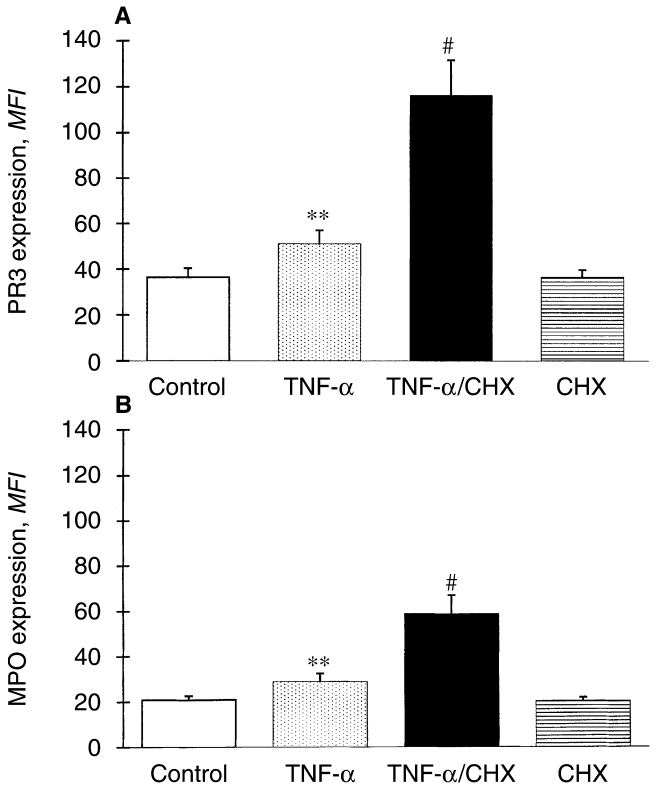

Fig. 5. Effects of apoptosis on ANCA antigen expression. PMN were cultured in buffer control, 10 ng/mL TNF-α (TNF-α), a combination of 10 ng/mL TNF-α and CHX (TNF-α/CHX), and CHX alone. Cells were assayed for proteinase-3 (PR3; *A*, $N = 13$) and myeloperoxidase (MPO; *B*, $N = 7$) membrane expression by flow cytometry. Ordinate shows membrane ANCA antigen expression as fluorescence intensity. Symbols are: (□) Control; (▩) TNF-α–treated cells; (■) TNF-α/CHX treatment; and (▤) CHX alone. TNF-α–mediated apoptosis was paralleled by a significantly increased expression of both PR3 and MPO. The accelerated apoptosis by TNF-α/CHX strongly augmented ANCA antigen expression (**$P < 0.01$ compared to Control; #$P < 0.01$ compared to all other groups).

generation in cells cultured in buffer, TNF-α, and TNF-α combined with cycloheximide, respectively. After 0, 1, 2, and 3 hours the cells were harvested, washed in HBSS and adjusted to 1×10^7/cells/mL. After priming with 2 ng/mL TNF-α, cells were stimulated with the mAb to MPO. Superoxide generation was measured in a continuous assay. Data are given for the representative 45-minute time point of activation (Fig. 7A). The increase in apoptosis was paralleled by a progressive loss of function in PMN brought to apoptosis with TNF-α. This effect was most pronounced in samples treated with a combination of TNF-α and cycloheximide. We performed a total of 10 experiments assessing the anti-MPO mAb-induced generation of superoxide after three hours of culture in buffer, TNF-α, TNF-α combined with cycloheximide (CHX), and cycloheximide alone, respectively. The data demonstrate that the three-hour TNF-α alone and combined treatment caused a decline in respiratory burst activity, whereas three hours of cycloheximide alone did not affect superoxide production (Fig. 7B).

The effects of TNF-α–induced apoptosis on the respiratory burst were examined using human ANCA preparations as stimuli. Figure 8 indicates that TNF-α–induced apoptosis also inhibited the amount of generated superoxide when PMN were challenged with human PR3-ANCA and human MPO-ANCA. We performed 11 experiments employing PR3-ANCA from three different donors, and 10 experiments employing MPO-ANCA from two different donors. The results confirm the observation from the experiments with the monoclonal anti-MPO antibody, in that TNF-α–mediated apoptosis decreased the amount of generated superoxide. Again, this effect was augmented when apoptosis was potentiated with the combined TNF-α + cycloheximide treatment for three hours. These data indicate that TNF-α and combined treatment-accelerated apoptosis is paralleled by a compromised ANCA-induced respiratory burst of PMN, despite an increase in ANCA-antigen expression. To test whether TNF-α-accelerated apoptosis also terminates the respiratory burst in response to other stimulatory agents, PMN were cultured in buffer, TNF-α, TNF-α + cycloheximide, and cycloheximide alone, respectively. After three hours, cells were harvested and stimulated with a receptor-independent activator, the

phorbol ester (PMA), a receptor-dependent stimulus, namely immune complexes, and bacterial peptide (FMLP). Figure 9 shows that accelerated apoptosis by TNF-α combined with cycloheximide significantly decreased the amount of generated superoxide in response to all of these activating substances.

Caspase-3 inhibition, ANCA antigen expression, and ANCA-induced respiratory burst

To investigate whether or not the observed changes in ANCA antigen expression and respiratory burst activity were causally related to apoptosis, we studied the effect of caspase-3 inhibition. PMN were cultured in control buffer or in TNF-α with cycloheximide combined treatment either with or without preincubation in 50 μmol/L of the specific caspase inhibitor Ac-DEVD-CMK. After three hours the cells were harvested and assayed for apoptosis, ANCA antigen expression, and superoxide generation. The specific inhibitor blocked TNF-α/CHX-mediated apoptosis almost completely as shown in Figure 10. As shown by propidium iodide-staining, the percentage of apoptotic PMN in the combined treatment sample decreased at three hours from 83 ± 2 to 2 ± 1% ($N = 8, P < 0.05$). Inhibition of apoptosis by Ac-DEVD-CMK was confirmed when cells were assayed using annexin V-staining (84 ± 6 to 7 ± 4%, $N = 2$). In addition, preincubation with Ac-DEVD-CMK also abrogated increased expression of PR3 and MPO in combined treatment samples. PR3 expression significantly decreased from 141.6 ± 34.1 to 33.9 ± 7.8 MFI (Fig. 11A), and MPO expression from 48.3 ± 12.9 to 11.9 ± 3.2 MFI (Fig. 11B). Finally, Ac-DEVD-CMK also prevented down-regulation of respiratory burst activity to the mAb to MPO (Fig. 12). PMN cultured in TNF-α plus cycloheximide in the absence of the caspase-3 inhibitor generated 17.0 ± 2.7 nmol $O_2^-/10^6$ PMN/45 min. This number was increased to 29.20 ± 3.4 nmol when Ac-DEVD-CMK was added to the culture ($N = 5, P < 0.05$). Ac-DEVD-CMK did not affect superoxide generation in control cells (32.6 ± 3.4 to 33.0 ± 2.5 nmol, $N = 5$, NS). These data indicate that caspase-dependent apoptosis was causative for both up-regulated ANCA antigen expression and the observed compromised respiratory burst to ANCA.

DISCUSSION

Polymorphonuclear neutrophils apoptosis is central for resolution of neutrophilic inflammation. We used a model of TNF-α–accelerated apoptosis to study the role of apoptosis on ANCA-induced PMN activation. Our data show that TNF-α–accelerated apoptosis significantly increased expression of ANCA antigens on the surface of PMN. However, despite increased ANCA-binding, apoptosis was paralleled by a progressive decrease in ANCA-induced respiratory burst. A causative relationship between apoptosis, increased ANCA antigen expression, and compromised respiratory burst was demonstrated by the fact that specific apoptosis inhibition with caspase-3 blockade prevented the increase in ANCA antigen expression and preserved the capability of generating superoxide.

Polymorphonuclear neutrophils activated by ANCA may be important to the neutrophilic inflammation in vasculitis. TNF-α is up-regulated in patients with active ANCA-associated vasculitis and its importance was demonstrated in experimental models of vasculitis [5–9]. TNF-α exhibits proinflammatory effects by priming PMN for ANCA-mediated activation in vitro [27–29]. However, TNF-α also accelerates PMN apoptosis [14–16]. The effect of TNF-α-apoptosis on ANCA-induced respiratory burst is not known. A suitable model to study this issue should induce apoptosis in the vast majority of cells without significant necrosis. In addition, short culture periods are wanted to reduce secondary effects associated with in vitro culture rather than with apoptosis. When we blocked TNF-α–induced survival signals with cycloheximide, apoptosis occurred rapidly, involving approximately 85% of the cells at three hours. We demonstrate that combined treatment with TNF-α and cycloheximide generated similar apoptotic cells, compared to TNF-α alone. Both conditions induced the typical nuclear condensation program as well as the cell surface program characteristic for apoptosis. Importantly, cell viability was above 95% as demonstrated by an observer-independent method. This finding suggests the absence of nuclear evanescence, since elegant studies by Hebert et al showed that nuclear evanescence is a sign of late apoptotic cells with high PI-fluorescence [30]. Thus, this model provides a good tool to study the role of TNF-α apoptosis in ANCA-induced activation.

We show that TNF-α–induced apoptosis was paralleled by an increased ANCA antigen expression. Double staining for apoptosis and ANCA antigen expression in TNF-α-treated samples demonstrated that TNF-α increased both PR3 and MPO on the surface of non-apoptotic PMN, as expected for TNF-α–mediated priming. However, TNF-α also induced apoptosis with an increase in the percentage of apoptotic cells that stained positive for ANCA antigens. Moreover, we observed a higher expression of the ANCA antigens on the surface of apoptotic cells, compared to the non-apoptotic population. Compared to TNF-α treatment alone, accelerated apoptosis by combined treatment with TNF-α and cycloheximide resulted in a further dramatic increase of PR3 and MPO expression, suggesting that apoptosis, in addition to the "classical" TNF-α priming, was responsible for this effect. In these samples, double staining revealed a further increase in the percentage of apoptotic cells that were positive for ANCA antigens. These data demon-

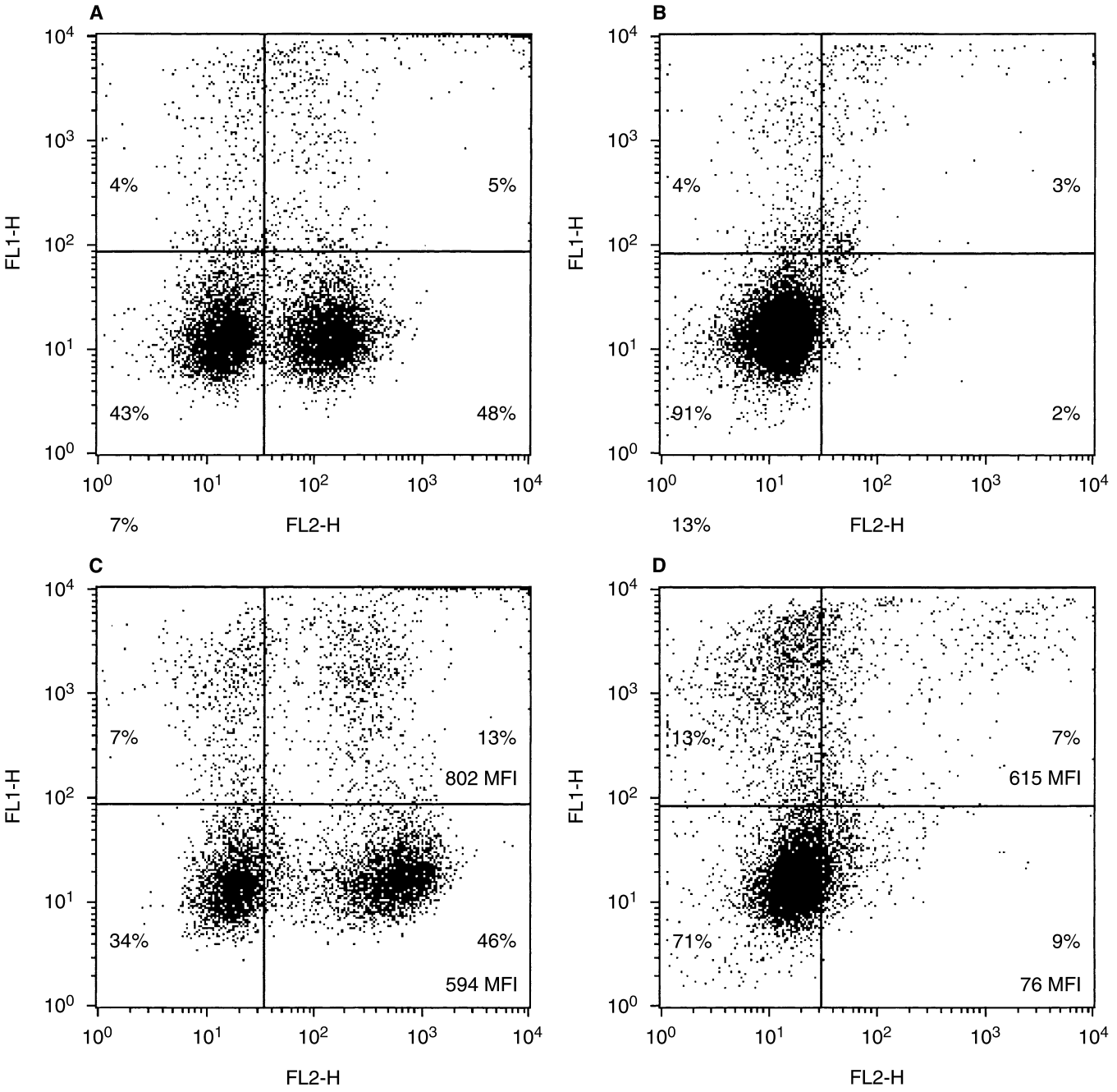

Fig. 6. Relationship between TNF-α–induced apoptosis and ANCA antigen expression. Cells were stained for apoptosis and either PR3 or MPO, respectively. FL1-H fluorescence shows apoptosis by annexin V binding and FL2-H fluorescence detects membrane staining for the ANCA antigens. Untreated controls are depicted in *A* (PR3 expression) and *B* (MPO expression). TNF-α increased ANCA antigens on the surface of non-apoptotic annexin V-negative PMN and triggered apoptosis with an increased percentage of apoptotic cells that stained positive for the ANCA antigens (upper right quadrants of *C* for PR3 and *D* for MPO), compared to untreated controls. Within the TNF-α–treated samples, apoptotic ANCA antigen-expressing cells (upper right quadrant) showed a higher MFI, compared to the non-apoptotic ANCA antigen-positive population (lower right quadrant).

strate that both TNF-α–mediated apoptosis and TNF-α–mediated priming increase the expression of ANCA-antigens. Our results are in agreement with the report by Gilligan et al, who demonstrated that constitutive PMN apoptosis was associated with translocation of PR3 and MPO from the cytoplasmic granules to the cell surface [18]. Our current study provides additional evidence for a role of apoptosis in increasing ANCA antigen expression in a model employing a cytokine that is up-regulated in ANCA-associated vasculitis. Increased ex-

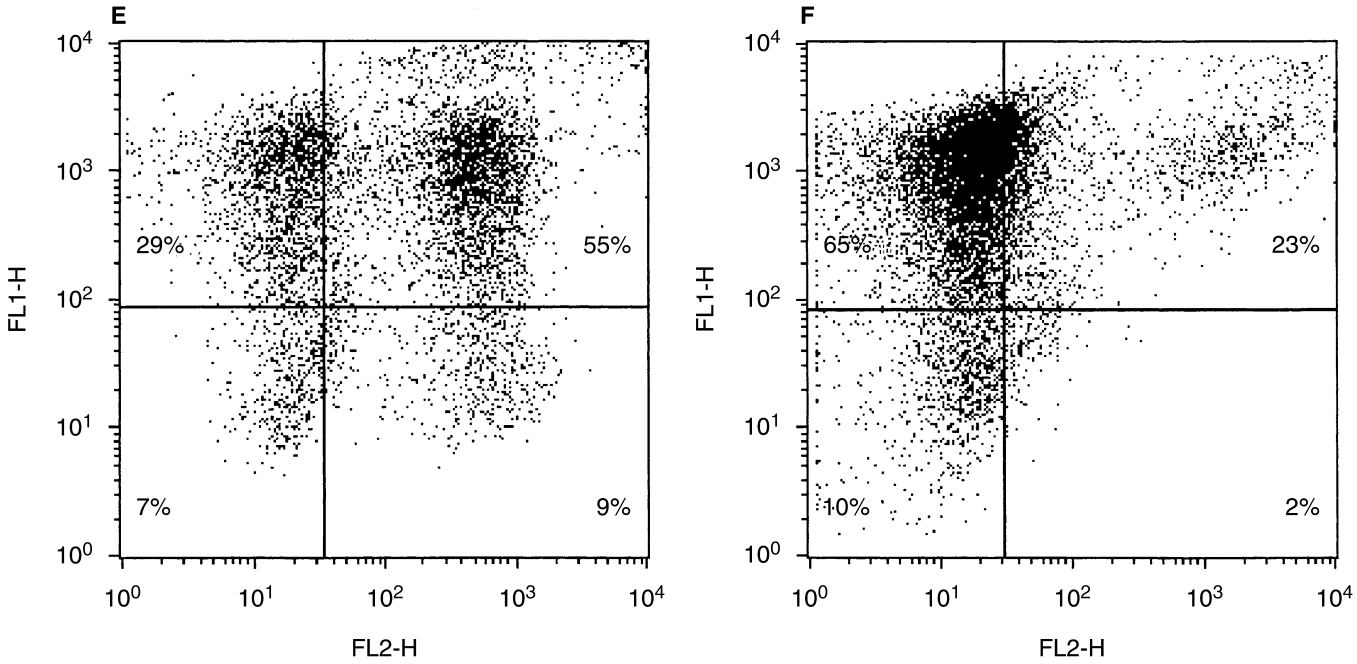

Fig. 6. (Continued) Combined treatment of TNF-α and cycloheximide (*E* for PR3 and *F* for MPO) resulted in a further increase of apoptotic cells that stained positive for the ANCA antigens (upper right quadrant). A representative experiment is depicted.

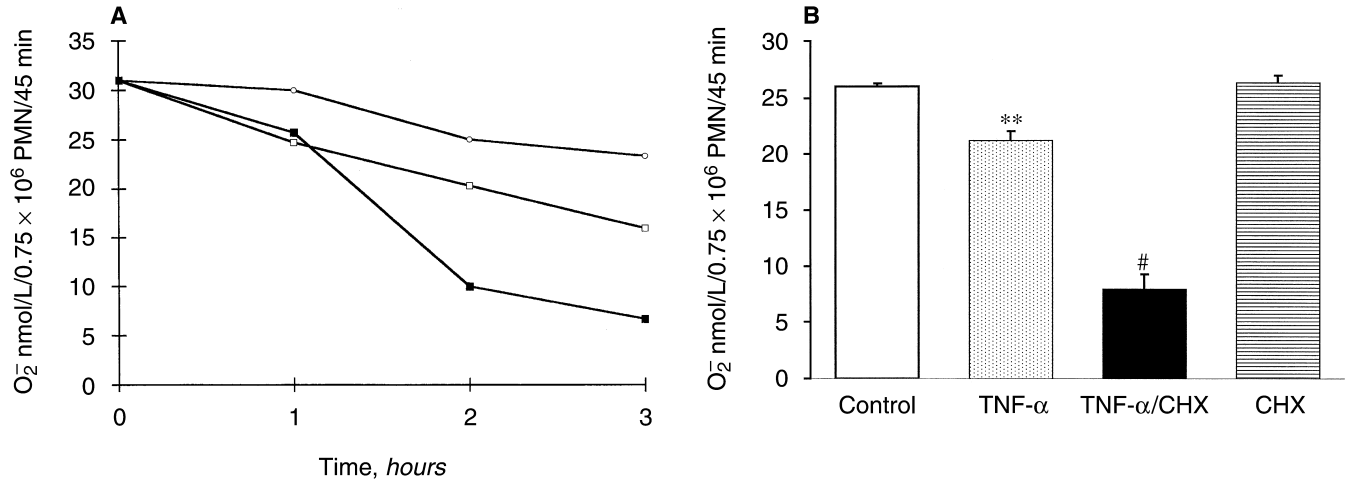

Fig. 7. Effect of TNF-α–mediated apoptosis on respiratory burst activity to an anti-MPO mAb. Superoxide dismutase-inhibitable reduction of ferricytochrome C was used. Data are given for the 45-minute time point. (*A*) Time course of the effect of controls (○), TNF-α (□) and TNF-α/CHX (■) on PMN superoxide generation in response to an anti-MPO mAb ($N = 3$). (*B*) Ten experiments were performed in parallel to assess the anti-MPO mAb-induced generation of PMN superoxide after 3 hours of culture in buffer (Control), TNF-α (TNF), TNF-α/CHX, and CHX, respectively. TNF-α–mediated apoptosis resulted in a significantly diminished respiratory burst to the anti-MPO mab (*$P < 0.01$ between TNF-α and Control). Potentiation of TNF-α–mediated apoptosis by CHX led to a further decrease in stimulated superoxide (#$P < 0.01$ compared to all other groups).

pression of PR3 is found in circulating PMN of patients with active Wegener's granulomatosis. High PR3 expression in isolated resting PMN, probably genetically determined, was recently identified as a risk factor for vasculitis by Wittko-Sarsat et al [31]. We observed a similar bimodal PR3 distribution as reported in their study. In the cohort of 126 healthy subjects described by Wittko-Sarsat et al, the size of the PR3 positive neutrophil population was stable in a given individual and ranged from 0 to 100%. Only 9% of the individuals had 0 to 20% PR3 positive neutrophils, 36% had 21 to 58% PR3 positive cells, and the majority of 55% had 59 to 100% PR3 positive neutrophils. This observation is very similar to our own donor population.

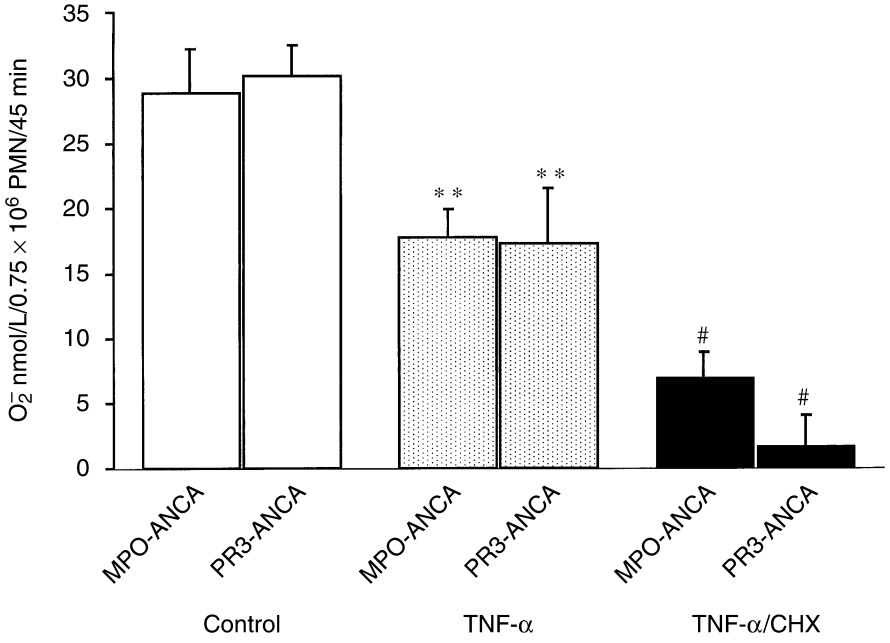

Fig. 8. Effects of TNF-α–mediated apoptosis on human ANCA-induced respiratory burst activity. PMN were harvested after 3 hours of culture in buffer (Control), TNF-α, TNF-α/CHX, respectively. Cells were stimulated with 75 μg/mL of human PR3-ANCA IgG from 3 different patients ($N = 11$) and 75 μg/mL MPO-ANCA IgG from 2 different patients ($N = 10$). Superoxide was measured by the ferricytochrome C assay and the 45-minute data are reported. These experiments indicate that TNF-α-mediated apoptosis significantly diminished respiratory burst in response to human ANCA (**$P < 0.01$ between TNF and Control). Potentiation of TNF-α-mediated apoptosis by CHX further decreased the amount of produced superoxide (#$P < 0.01$ compared to all other groups).

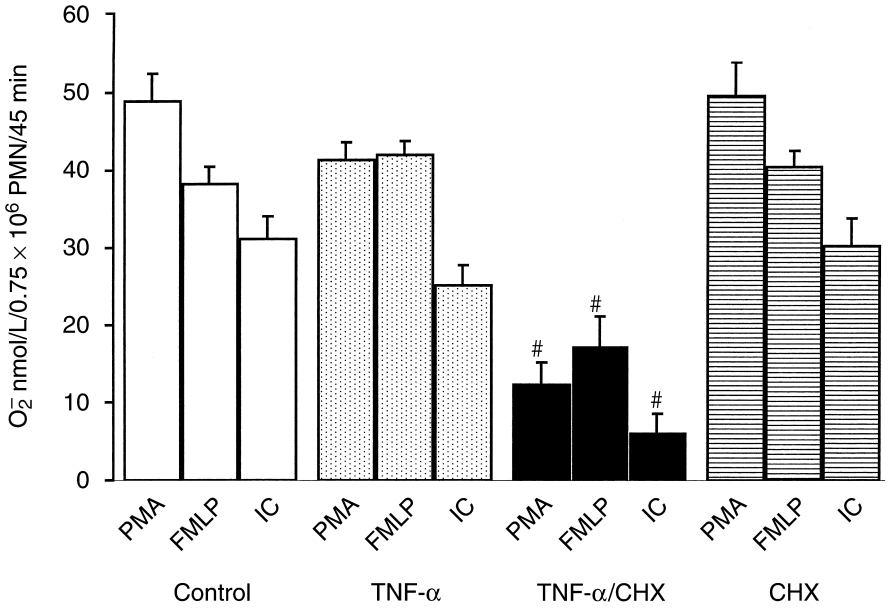

Fig. 9. Effects of TNF-α–mediated apoptosis on superoxide generation in response to PMA, immune-complexes, and FMLP was studied employing the ferricytochrome C assay. PMN were harvested after 3 hours of culture in buffer (Control), TNF-α, TNF-α/CHX, and CHX, respectively. Cells were stimulated with 25 ng/mL PMA ($N = 8$), 25 μg/mL immune complexes ($N = 8$), and 10^{-7} mol/L FMLP ($N = 9$), respectively. The data indicate that apoptosis by combined treatment with TNF-α and CHX significantly decreased the amount of produced superoxide (#$P < 0.01$ compared to all other groups).

Whether or not increased ANCA antigen expression by apoptosis allows more ANCA-neutrophil interaction, and thereby prevents or overrides potential activation-limiting signals of apoptosis, is not yet known. Our data show that TNF-α–accelerated apoptosis was paralleled by a compromised respiratory burst to human ANCA and to the mAb to MPO. This effect occurred in a time-dependent fashion and was not caused by cell necrosis. Thus, apoptosis by TNF-α results in the loss of respiratory burst activity to ANCA that was not prevented by increased ANCA binding. Also, apoptotic cells stimulated with PMA, FMLP, and immune complexes showed decreased superoxide generation. Remarkably, the amount of generated superoxide to all these stimuli was significantly reduced but not completely abrogated. Thus, apoptotic PMN that have not yet progressed into necrosis may still provide a source of reactive oxygen species. Previous studies have suggested that the effect of apoptosis on neutrophil function depends on the way apoptosis is induced as well as on the agent used to stimulate cells. In a model of constitutive apoptosis two studies have shown that PMN apoptosis is associated

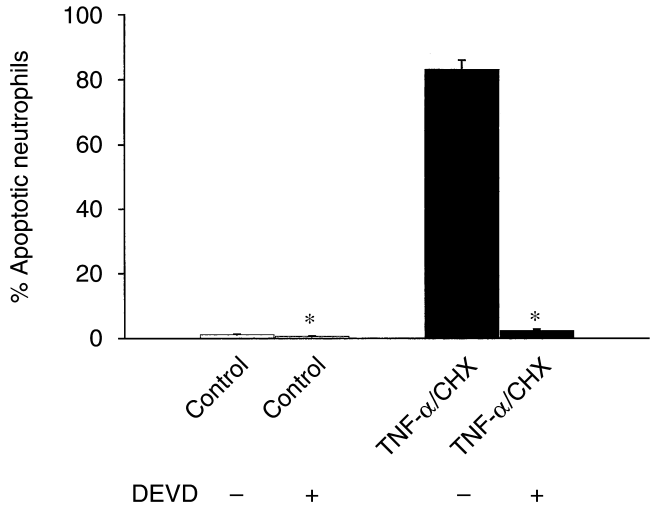

Fig. 10. Effects of caspase inhibition on TNF-α–mediated apoptosis. PMN were cultured for 3 hours in buffer (Control) or TNF-α/CHX in the presence or absence of 50 μmol/L Ac-DEVD-CMK. Samples were harvested and the percentage of apoptotic cells was determined by flow cytometry. The data show that inhibition of caspase-3 almost completely blocked apoptosis in cells treated with TNF-α/CHX ($N = 6$, $^*P < 0.05$).

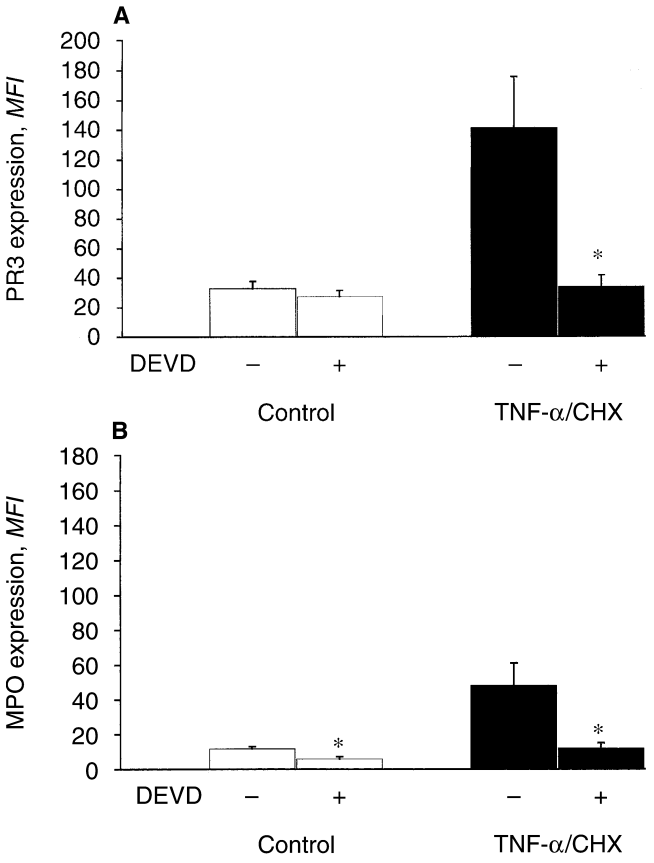

Fig. 11. Effects of caspase inhibition on ANCA antigen expression. PMN were cultured for 3 hours in buffer (Control) or TNF-α/CHX in the presence or absence of 50 μmol/L Ac-DEVD-CMK. Samples were harvested and membrane expression of PR3 (*A*) and MPO (*B*) was assessed by flow cytometry ($N = 5$). These experiments indicate that blocking caspase activity prevents up-regulation of ANCA antigens ($^*P < 0.05$).

with a reduced capability to generate superoxide in response to receptor-mediated activators, whereas receptor-independent stimulation with PMA was not [17, 32]. In a TNF-α–induced apoptosis model Yamashita et al demonstrated that apoptosis-associated down-regulation of reactive oxygen production to PMA and opsonized zymosan [33]. Our results are in agreement with their data showing diminished superoxide generation in apoptotic cells activated by PMA and in addition also to two receptor-dependent stimuli, such as immune complexes and the bacterial peptide FMLP. Whether ANCA bind to their specific antigens via a receptor or simply by charge is not yet known. Interestingly, Taekema Roelvink et al recently characterized a 111 kD molecule that may function as a PR3-presenting receptor in endothelial cells [34]. However, the existence of such a receptor in human PMN must be established.

Caspases are important mediators of apoptosis. We and others have shown that active caspases, including caspase-3, are important for TNF-α–mediated neutrophil apoptosis [33, 35, 36]. Our current study used caspase inhibition to examine the causal relationship between TNF-α–accelerated apoptosis, increased ANCA antigen expression, and loss of respiratory burst activity to ANCA. The cell-permeable compound Ac-DEVD-CMK inhibits preferentially the executioner caspase-3. Preincubation with Ac-DEVD-CMK blocked accelerated apoptosis and prevented both the increase in ANCA antigen expression as well as the loss of respiratory burst activity to ANCA. These experiments suggest that apoptosis is indeed the cause for both effects.

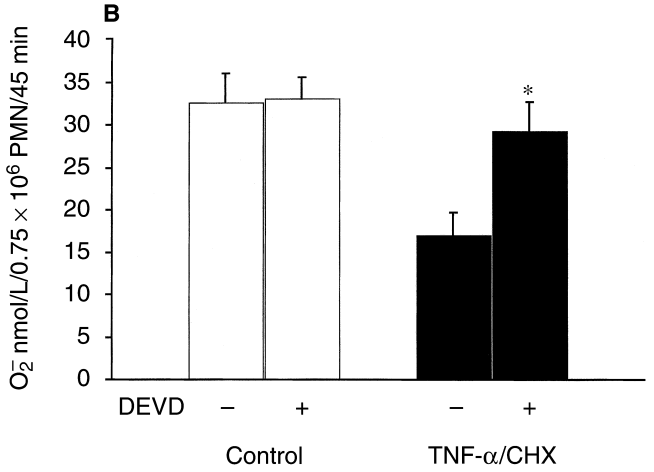

Fig. 12. Effects of caspase inhibition on respiratory burst. PMN were cultured in buffer (Control) or TNF-α/CHX in the presence or absence of 50 μmol/L Ac-DEVD-CMK. After 3 hours cells were harvested and superoxide generation to a mAb to MPO was determined by ferricytochrome C reduction ($N = 5$). These experiments demonstrate that inhibition of caspase-3 preserved respiratory burst activity ($^*P < 0.05$).

In conclusion, we analyzed the effect of TNF-α–mediated apoptosis on ANCA-induced PMN respiratory burst. Our data indicate that TNF-α–accelerated apoptosis is associated with increased ANCA antigen expression, providing more binding of ANCA. However, the respiratory burst in apoptotic PMN to ANCA was progressively compromised. A causative relationship was suggested, since specific inhibition of apoptosis by blocking caspase activity inhibited ANCA antigen up-regulation and prevented the decrease in ANCA-induced superoxide generation. TNF-α is increased in active vasculitis. Our data suggest that TNF-α exhibits dual function by the well-known priming effect on ANCA-mediated PMN activation and also by limiting ANCA-induced activation, as shown here for the respiratory burst. Accelerating apoptosis by medical treatment may be helpful in patients with ANCA-mediated vasculitis.

Reprint requests to Ralph Kettritz, M.D., Division of Nephrology, Franz Volhard Clinic, Wiltbergstrasse 50, 13122 Berlin, Germany. E-mail: kettritz@fvk-berlin.de

REFERENCES

1. van der Woude FJ, Rasmussen N, Lobatto S, *et al*: Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker for disease activity in Wegener's granulomatosis. *Lancet* i:425–429, 1985
2. Falk RJ, Jennette JC: Anti-neutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis. *N Engl J Med* 318:1651–1657, 1988
3. Kallenberg CG, Brouwer E, Weening JJ, Tervaert JW: Anti-neutrophil cytoplasmic antibodies: Current diagnostic and pathophysiological potential. *Kidney Int* 46:1–15, 1994
4. Savage CO, Winearls CG, Jones S, *et al*: Prospective study of radioimmunoassay for antibodies against neutrophil cytoplasm in diagnosis of systemic vasculitis. *Lancet* 1:1389–1393, 1987
5. Noronha IL, Kruger C, Andrassy K, *et al*: In situ production of TNF-alpha, IL-1 beta and IL-2R in ANCA-positive glomerulonephritis. *Kidney Int* 43:682–692, 1993
6. Watanabe N, Akikusa B, Park SY, *et al*: Mast cells induce autoantibody-mediated vasculitis syndrome through tumor necrosis factor production upon triggering Fcgamma receptors. *Blood* 94:3855–3863, 1999
7. Tomer Y, Barak V, Gilburd B, Shoenfeld Y: Cytokines in experimental autoimmune vasculitis: Evidence for a Th2 type response. *Clin Exp Rheumatol* 17:521–526, 1999
8. Kuryliszyn Moskal A: Cytokines and soluble CD4 and CD8 molecules in rheumatoid arthritis: Relationship to systematic vasculitis and microvascular capillaroscopic abnormalities. *Clin Rheumatol* 17:489–495, 1998
9. Ludviksson BR, Sneller MC, Chua KS, *et al*: Active Wegener's granulomatosis is associated with HLA-DR+ CD4+ T cells exhibiting an unbalanced Th1-type T cell cytokine pattern: Reversal with IL-10. *J Immunol* 160:3602–3609, 1998
10. Falk RJ, Terrell RS, Charles LA, Jennette JC: Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro. *Proc Natl Acad Sci USA* 87:4115–4119, 1990
11. Mulder AH, Heeringa P, Brouwer E, *et al*: Activation of granulocytes by anti-neutrophil cytoplasmic antibodies (ANCA): A Fc gamma RII-dependent process. *Clin Exp Immunol* 98:270–278, 1994
12. Porges AJ, Redecha PB, Kimberly WT, *et al*: Anti-neutrophil cytoplasmic antibodies engage and activate human neutrophils via Fc gamma RIIa. *J Immunol* 153:1271–1280, 1994
13. Kettritz R, Schreiber A, Luft FC, Haller H: Role of mitogen-activated protein kinases in activation of human neutrophils by antineutrophil cytoplasmic antibodies. *J Am Soc Nephrol* 12:37–46, 2001
14. Takeda Y, Watanabe H, Yonehara S, *et al*: Rapid acceleration of neutrophil apoptosis by tumor necrosis factor-alpha. *Int Immunol* 5:691–694, 1993
15. Kettritz R, Falk RJ, Jennette JC, Gaido ML: Neutrophil superoxide release is required for spontaneous and FMLP-mediated but not for TNF alpha-mediated apoptosis. *J Am Soc Nephrol* 8:1091–1100, 1997
16. Murray J, Barbara JA, Dunkley SA, *et al*: Regulation of neutrophil apoptosis by tumor necrosis factor-alpha: Requirement for TNFR55 and TNFR75 for induction of apoptosis in vitro. *Blood* 90:2772–2783, 1997
17. Whyte MK, Meagher LC, MacDermot J, Haslett C: Impairment of function in aging neutrophils is associated with apoptosis. *J Immunol* 150:5124–5134, 1993
18. Gilligan HM, Bredy B, Brady HR, *et al*: Antineutrophil cytoplasmic autoantibodies interact with primary granule constituents on the surface of apoptotic neutrophils in the absence of neutrophil priming. *J Exp Med* 184:2231–2241, 1996
19. Wang CY, Mayo MW, Baldwin AS Jr: TNF- and cancer therapy-induced apoptosis: Potentiation by inhibition of NF-kappaB. *Science* 274:784–787, 1996
20. Van Antwerp DJ, Martin SJ, Verma IM, Green DR: Inhibition of TNF-induced apoptosis by NF-kappa B. *Trends Cell Biol* 8:107–111, 1998
21. Kettritz R, Jennette JC, Falk RJ: Crosslinking of ANCA-antigens stimulates superoxide release by human neutrophils. *J Am Soc Nephrol* 8:386–394, 1997
22. Kerr JF, Wyllie AH, Currie AR: Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 26:239–257, 1972
23. Pick E, Mizel D: Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. *J Immunol Methods* 46:211–226, 1981
24. Salmon JE, Cronstein BN: Fc gamma receptor-mediated functions in neutrophils are modulated by adenosine receptor occupancy. A1 receptors are stimulatory and A2 receptors are inhibitory. *J Immunol* 145:2235–2240, 1990
25. Lin WW, Hsu YW: Cycloheximide-induced cPLA(2) activation is via the MKP-1 down-regulation and ERK activation. *Cell Signal* 12:457–461, 2000
26. Yang GH, Jarvis BB, Chung YJ, Pestka JJ: Apoptosis induction by the satratoxins and other trichothecene mycotoxins: Relationship to ERK, p38 MAPK, and SAPK/JNK activation. *Toxicol Appl Pharmacol* 164:149–160, 2000
27. Charles LA, Caldas ML, Falk RJ, *et al*: Antibodies against granule proteins activate neutrophils in vitro. *J Leukoc Biol* 50:539–546, 1991
28. Savage CO, Pottinger BE, Gaskin G, *et al*: Autoantibodies developing to myeloperoxidase and proteinase 3 in systemic vasculitis stimulate neutrophil cytotoxicity toward cultured endothelial cells. *Am J Pathol* 141:335–342, 1992
29. Reumaux D, Vossebeld PJ, Roos D, Verhoeven AJ: Effect of tumor necrosis factor-induced integrin activation on Fc gamma receptor II-mediated signal transduction: relevance for activation of neutrophils by anti-proteinase 3 or anti-myeloperoxidase antibodies. *Blood* 86:3189–3195, 1995
30. Hebert MJ, Takano T, Holthofer H, Brady HR: Sequential morphologic events during apoptosis of human neutrophils. Modulation by lipoxygenase-derived eicosanoids. *J Immunol* 157:3105–3115, 1996
31. Witko Sarsat V, Lesavre P, Lopez S, *et al*: A large subset of neutrophils expressing membrane proteinase 3 is a risk factor for vasculitis and rheumatoid arthritis. *J Am Soc Nephrol* 10:1224–1233, 1999
32. Cendoroglo M, Jaber BL, Balakrishnan VS, *et al*: Neutrophil

apoptosis and dysfunction in uremia. *J Am Soc Nephrol* 10:93–100, 1999

33. Yamashita K, Takahashi A, Kobayashi S, *et al*: Caspases mediate tumor necrosis factor-alpha-induced neutrophil apoptosis and downregulation of reactive oxygen production. *Blood* 93:674–685, 1999
34. Taekema Roelvink ME, Van Kooten C, Heemskerk E, *et al*: Proteinase 3 interacts with a 111-kD membrane molecule of human umbilical vein endothelial cells. *J Am Soc Nephrol* 11:640–648, 2000
35. Watson RW, Rotstein OD, Parodo J, *et al*: Impaired apoptotic death signaling in inflammatory lung neutrophils is associated with decreased expression of interleukin-1 beta converting enzyme family proteases (caspases). *Surgery* 122:163–171, 1997
36. Kettritz R, Xu YX, Faass B, *et al*: TNF-alpha-mediated neutrophil apoptosis involves Ly-GDI, a Rho GTPase regulator. *J Leukoc Biol* 68:277–283 (Issn: 0741–5400, 2000)